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Roles for RAB24 in autophagy and disease

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Abbreviations

GABARAP, gamma-aminobutyric acid receptor-associated protein

GAP, GTPase activating protein

GDF, GDI displacement factor

GDI, GDP dissociation inhibitor

GEF, GDP-GTP exchange factor

GOSR1, SNARE protein Golgi SNAP receptor complex member 1

HCC, hepatocellular carcinoma

Hsc70, heat shock cognate protein of 70 kDa

LAMP2A, lysosomal associated membrane protein type 2A

LC3, microtubule-associated protein light chain 3

mTOR, mammalian target of rapamycin

NSF, N-ethylmaleimide sensitive fusion protein

RILP, RAB7 interacting lysosomal protein

SNAP29, synaptosomal associated proten 29

SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor

Abstract

Autophagy is an evolutionarily conserved degradation pathway for cells to maintain homeostasis, produce energy, degrade misfolded proteins and damaged organelles, and fight against intracellular pathogens. The process of autophagy entails the isolation of cytoplasmic cargo into double membrane bound autophagosomes that undergo maturation by fusion with endosomes and lysosomes in order to obtain degradation capacity. RAB proteins regulate intracellular vesicle trafficking events including autophagy. RAB24 is an atypical RAB protein that is required for the clearance of late autophagic vacuoles under basal conditions. RAB24 has also been connected to several diseases including ataxia, cancer and tuberculosis. This review gives a short summary on autophagy and RAB proteins, and an overview on the current knowledge on the roles of RAB24 in autophagy and disease.

RAB proteins in membrane trafficking

RAB proteins regulate all steps in intracellular membrane dynamics such as cargo selection, vesicle budding and transport along cytoskeletal tracks, as well as vesicle docking and fusion.[1, 2] RABs are synthesized as soluble proteins that are post-translationally modified by the covalent attachment of a geranylgeranyl moiety, also called a prenyl group, to their C-terminal cysteines, which enables their association on the cytosolic side of intracellular membranes.[3] RAB proteins cycle between

active GTP- and membrane- bound state, and inactive GDP-bound cytosolic state. Inactive GDP-bound RABs can be activated on membrane surfaces by the action of GDP-GTP exchange factors (GEFs).[4] While in the active GTP-bound state, RAB proteins are able to recruit effectors that function in the different vesicular trafficking steps. The GTPase activity of RABs is controlled by the GTPase activating proteins (GAPs). GTP hydrolysis leads to the inactivation of the RAB and subsequently, RAB-GDP re-associates with GDP dissociation inhibitors (GDIs) and is retrieved from the membrane. GDIs hide the hydrophobic prenyl groups in their hydrophobic groove, making the RABs soluble in the cytoplasm.[5] Dissociation of RAB-GDP from GDI, and subsequent membrane insertion, are achieved by the action of a GDI displacement factor (GDF).[6] Unlike GDIs, GEFs and GAPs show more specificity for their target RABs.[4] The RAB activation/inactivation cycle is schematically presented in Figure 1.

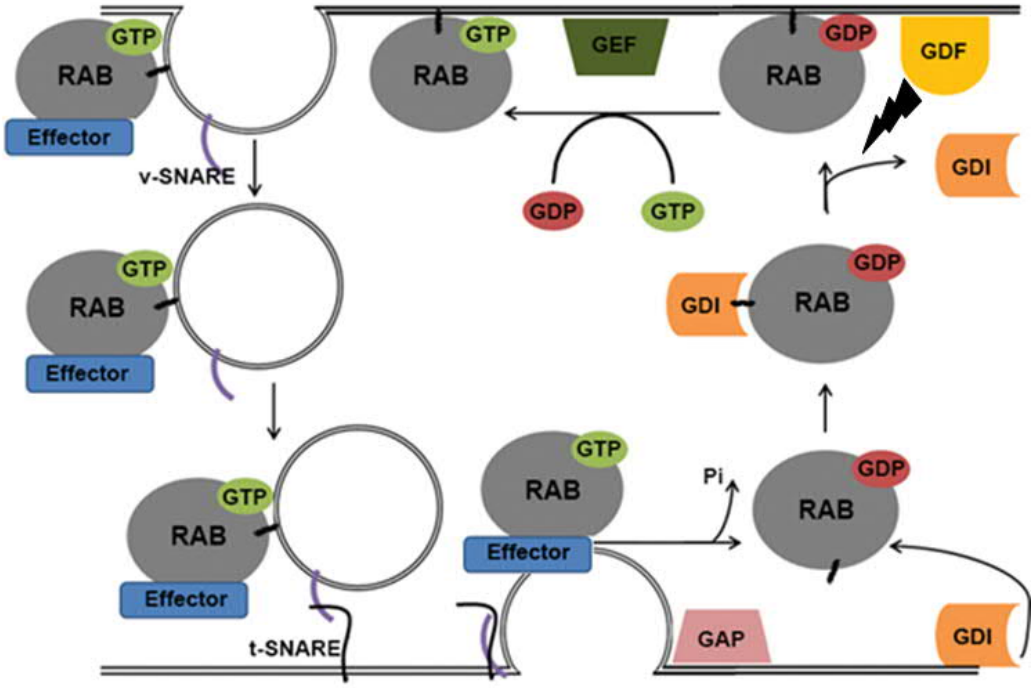

Figure 1. The RAB activation/inactivation cycle. RAB proteins cycle between active membrane bound state and inactive cytosolic state. RABs recruit effector proteins while in the active GTP-bound state (left). See text for further details. GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor; Pi, inorganic phosphate; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; t-SNARE, SNARE on target membrane; v-SNARE, SNARE on vesicle membrane.

RAB effectors interact with the active, GTP-bound form of their partner RAB proteins and mediate at least one specific downstream effect. RAB effectors belong to many different protein families and mediate a wide selection of functions including the selection and concentration of vesicle cargo, vesicle formation, vesicle transport along actin filaments or microtubules, as well as vesicle recognition and fusion. Many RAB effectors serve a tethering function linking opposing membranes before SNARE pairing.[7] One RAB can interact with several different effector proteins.[8]

Autophagy

Autophagy is an evolutionarily conserved cellular waste disposal and recycling mechanism, where cytoplasmic components are transported to lysosomes for degradation. Autophagy helps the cells to maintain homeostasis by producing energy and building blocks for vital biosynthetic reactions, degrading misfolded and aggregated proteins and unnecessary organelles, and fighting against intracellular pathogens.[9, 10] There are three ways to transport cytoplasmic material to lysosomes, called macroautophagy (or simply autophagy), microautophagy and chaperone-mediated autophagy (Figure 2). Macroautophagy involves the formation of an autophagosome, i.e., the enwrapping of the cytoplasmic cargo into a double membraned vacuole, and the subsequent delivery of the sequestered material for degradation by fusion with endosomes and lysosomes. Macroautophagy is able to degrade cytosolic proteins, ribosomes, protein aggregates and whole organelles. Microautophagy occurs by direct inward budding of the lysosomal limiting membrane with the engulfed cargo.[11] Chaperone-mediated autophagy is a specific transport route through the lysosomal membrane where the cargo protein must contain a recognition motif (KFERQ) that is recognized by a cytosolic chaperone, heat shock cognate protein of 70 kDa (Hsc70). This complex binds to the lysosomal receptor protein called lysosomal associated membrane protein type 2A (LAMP2A).[12] After unfolding, the cargo protein is transported across the lysosomal membrane with the help of the chaperone Hsc70. After degradation of autophagic substrates, the degradation products are transported back to the cytoplasm through several lysosomal permeases.

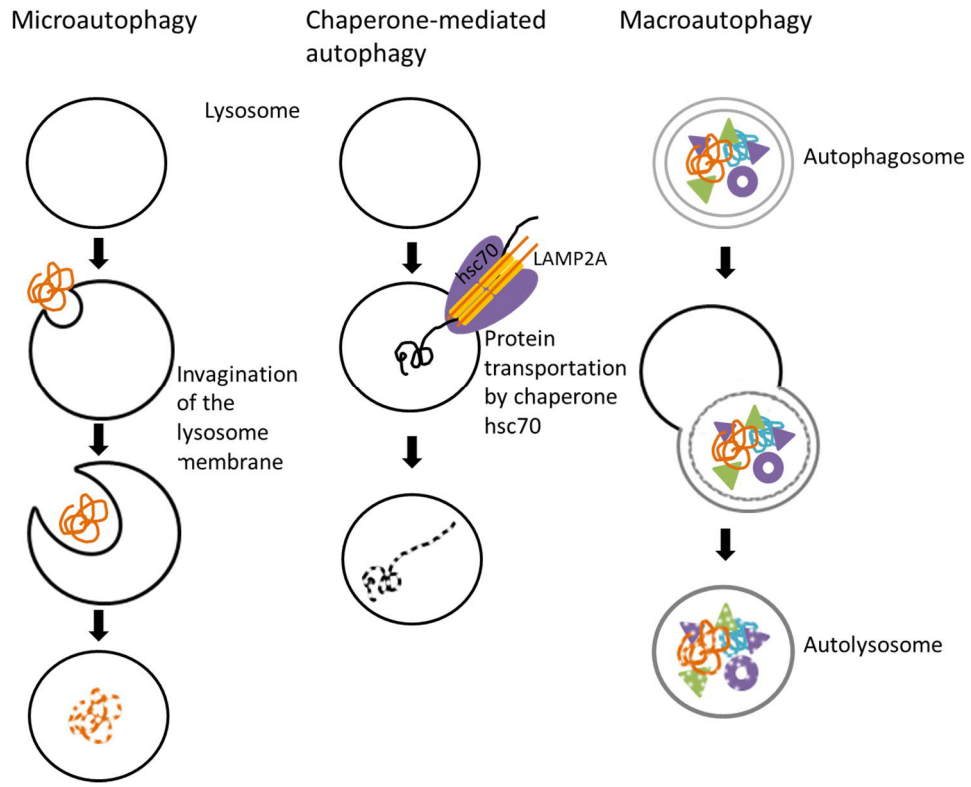

Figure 2. There are three types of autophagy: microautophagy, chaperone-mediated autophagy and macroautophagy. See text for further details.

Autophagy is induced by different stimuli including stress and amino acid starvation, but autophagy also exists when nutrients are available. The non-induced or basal autophagy enforces intracellular quality control and is of particular importance for postmitotic cells like neurons and muscle cells. Basal autophagy occurs continuously at a low level and degrades old organelles and aggregate-prone proteins; common denominators in age-related disorders such as neurodegenerative diseases. Starvation-induced autophagy and basal autophagy seem to differ in substrate selectivity and in regulation. While starvation-induced autophagy is inhibited by the target of rapamycin (mTOR) kinase, basal autophagy is less affected by mTOR.[13] Notably, when mTOR activation is caused by overexpression of RHEB or activated RAGs (as opposed to activation by the presence of nutrients), also basal autophagy is suppressed.[14] Further, basal autophagy is less dependent on phosphatidyl inositol 3-kinase activity than induced autophagy.[14] The maturation of basal and starvation-induced autophagosomes also differs. One example is RAB7 that functions during the maturation of starvation-induced autophagosomes but seems to be dispensable for basal autophagy.[15]

Autophagic degradation requires several membrane fusion events, and not surprisingly, many RAB proteins and other small GTPases have been described to function in autophagy. Some GTP binding proteins function in autophagosome induction or formation (RAB1B, RAB4, RAB5, RAB11, RAB32, RAB33B, and SAR1)[16-21] and others later in the lysosomal fusion processes (RAB7, RAB8B, RAB11, RAB24 and RAB33B).[15, 22-25] RAB7 is also needed for the formation of autophagosomes induced by intracellular Streptococcus bacteria.[26] RAB9 is required for an unconventional form of macroautophagy that is independent of ATG5 and ATG7 autophagy proteins.[27] RAB8A plays a role in the unconventional autophagic secretory pathway for interleukin 1b.[28] RAB39A and RAB25 have been shown to negatively regulate autophagy.[29, 30] The roles of RAB GTPases and their regulators in autophagy have been summarized in several recent reviews.[31-37]

RAB24 is an atypical RAB protein that has been implicated in autophagy for a long time. Recent research has finally demonstrated that RAB24 is required in basal autophagy, and shown that RAB24 may be connected to several diseases. In this review, we summarize what is currently known about the roles of RAB24 in macroautophagy and disease.

RAB24 is an unusual RAB protein

Elias et al. performed a genomics analysis on the evolutionary history of RAB proteins.[38] This analysis places RAB24 among the primordial RABs that were present in the last eukaryotic common ancestor. RAB24 was proposed to be one of the RABs that associate with the establishment of the endocytic pathway in eukaryotic cells. RAB24 is conserved in many species including Dictyostelium discoideum, zebrafish and mammals, but has been lost in others including Saccharomyces cerevisiae, Drosophila melanogaster and Caenorhabditis elegans.

RAB24 protein was first characterized by Olkkonen et al. as a perinuclear protein that colocalizes with Golgi markers, some late endosome markers, and with RAB2, an ER-Golgi intermediate compartment marker.[39] Later, RAB24 was reported to differ from a typical RAB protein in several aspects. Erdman et al. found that RAB24 has a low GTPase activity and thus predominantly occurs in the GTP-bound state. They also reported that RAB24 is inefficiently prenylated and that cytosolic

148 RAB24 only weakly associates with GDI.[40] Later studies have shown conflicting results on the GDI
149 binding and prenylation. Using immunoprecipitation, Behrends et al. reported that RAB24 interacts
150 with both GDI1 and GDI2.[41] Further, we showed that overexpressed RAB24 and RAB7 are prenylated
151 at similar extents.[42] Ding et al. observed that the cytosolic pool of RAB24 is more phosphorylated
152 than the membrane-associated pool.[43] Two tyrosines were found to be phosphorylated, Y17 within
153 the GXXXGK(S/T) motif known as the P-loop, and Y172 in the YXXf motif in the hypervariable
154 domain. The low GTPase activity of RAB24 is thought to be associated with the unusual amino acid
155 at position 67 in the GTP-binding region: this amino acid is serine in RAB24, while in other RABs
156 residue 67, or its equivalent, in the GTP-binding region is glutamine. The P-loop containing tyrosine
157 Y17 may also influence GTP hydrolysis.[43] In other RAB proteins, Q67L or equivalent mutation causes
158 a constitutively active RAB phenotype. However, RAB24-S67L mutant binds GTP less efficiently than
159 wild type RAB24, does not localize to any membranous organelles, and acts as a dominant negative
160 mutant when overexpressed in cells.[42, 44, 45]

161

162 RAB24 and autophagy

163 Already Olkkonen et al. suggested that RAB24 may function in some sort of autophagy-related
164 transport route between the ER-Golgi intermediate compartment and late endocytic
165 compartments.[39] Later, several laboratories published reports supporting a role for RAB24 in
166 autophagy. Munafo and Colombo showed that overexpressed RAB24 changes localization upon
167 amino acid starvation.[46] In starved cells, RAB24 colocalized in vesicular structures with the
168 autophagosome marker LC3 (microtubule-associated protein light chain 3) and with
169 monodansylcadaverine, a marker for acidic compartments and putative autolysosomes. The same
170 group observed that transit through the autophagic pathway increased the infection of CHO cells
171 with Coxiella. Overexpression of wild type RAB24 accelerated, while expression of mutant RAB24-
172 S67L inhibited the formation of Coxiella vacuoles.[44] Wu et al. observed that RAB24 colocalized with
173 another autophagosome marker protein called GABARAP.[47] Tambe et al. reported that the tumor
174 suppressor protein DRS is involved in autophagosome maturation.[48] They also showed
175 colocalization of DRS and RAB24, and of DRS and the autophagosome marker LC3, in punctate
176 structures that accumulated in low serum culture conditions. Marambio et al. studied aggresome
177 formation in cultured cardiac myocytes exposed to glucose deprivation.[49] Aggresomes form when
178 proteasomal degradation is overwhelmed with aggregate-prone proteins. Both LC3 and RAB24

colocalized with the aggresomes. Taken together, all these findings provided evidence for RAB24 function in autophagy, but none of these studies addressed whether RAB24 was actually required for autophagy, and thus the exact step where RAB24 would function also remained unclear.

RAB24 has also been observed to be upregulated during cellular stress, which is also known to induce autophagy. Egami et al. reported that RAB24 mRNA levels increased in nerve-injured hypoglossal motor neurons of rats.[50] Similar increase in RAB24 mRNA was observed in differentiated PC12 cells treated with a proteasomal inhibitor. Also mRNA levels of LC3 increased in both hypoglossal motor neurons and PC12 cells. Further, RAB24 showed partial colocalization with LC3 in immunofluorescence staining. Similar increase in RAB24 protein level was reported by Seki et al. in trigeminal motor nucleus after denervation.[51]

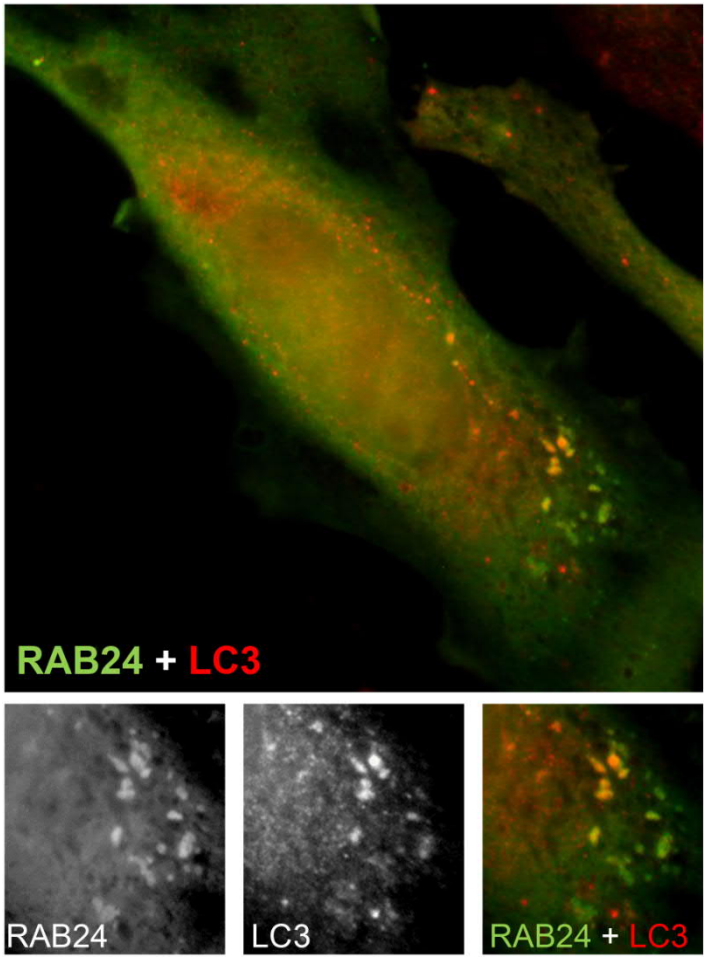

Figure 3. RAB24 colocalizes with the autophagosome marker LC3. HeLa cells were transfected with RAB24 and immunolabeled with anti-RAB24 and anti-LC3. Before fixation, the cells were treated with 100 mM leupeptin and 10 mg/ml pepstatin for 6 h in full culture medium in order to accumulate

autophagic vacuoles (autophagosomes, amphisomes and autolysosomes) under basal conditions. Yellow color in the overlay images indicates colocalization.

RAB24 plays a role in basal autophagy and endosomal degradation

We studied the role of RAB24 in macroautophagy using HeLa and NRK cells.[42] We observed RAB24 to colocalize with approximately 60% of LC3-positive autophagic structures both under basal and starvation conditions (Figure 3). Although the percentage of LC3-positive structures also positive for RAB24 did not increase during amino acid starvation, the amount of RAB24 per LC3 vesicle did increase. Immuno electron microscopy showed RAB24 to localize to both the inner and outer limiting membranes of autophagosomes. Using subcellular fractionation, we further showed that endogenous RAB24 localized to fractions positive for LC3-II, the membrane-associated form of LC3, and SQSTM1, an autophagic cargo protein. We also showed that targeting of RAB24 to autophagosomes requires prenylation and GTP binding, but not phosphorylation of tyrosines Y17 or Y172. Our results further showed that RAB24 is dispensable for the formation, maturation and clearance of starvation-induced autophagosomes. However, under basal conditions, acidic autolysosomes accumulated in RAB24-depleted cells. Using bafilomycin to inhibit autophagic flux, we showed the accumulation to be due to decreased clearance of autolysosomes. We also showed that depletion of RAB24 retarded the clearance of a Huntingtin-polyglutamine probe, which has been shown to be a substrate for autophagic clearance.[13] Finally, we observed that the degradation of long-lived proteins was slightly decreased under basal conditions in RAB24 silenced cells.[42] We concluded that RAB24 functions in the clearance of autolysosomes under basal conditions, but is not needed for starvation-induced autophagy. Thus RAB24 is the first RAB protein shown to be required in the very late steps of basal autophagy. Two pathways have been described for autolysosome clearance: reformation of lysosomes from autolysosomes,[52] and fusion of autolysosomes with the plasma membrane.[53] It remains to be shown whether RAB24 plays a role in these processes.

A recent study by Amaya et al.[54] showed that RAB24 coprecipitated with the late endosomal/lysosomal RAB7 and its effector RILP (RAB7 interacting lysosomal protein). As mentioned earlier, RAB7 is also needed for the fusion of starvation-induced autophagosomes with

lysosomes.[15, 22] RAB24 was shown to colocalize with RAB7, and the localization of RAB7 to vesicular structures was shown to require RAB24. [54] Further, RAB24 was found to be needed for the degradation of endocytic cargo. The authors concluded that RAB24 forms a complex with RAB7 and RILP on the surface of late endosomal/lysosomal compartments and regulates endosomal degradation. Thus, two recent studies[42, 54] indicate that RAB24 functions in the late stages of autophagic and endocytic pathways.

RAB24 associates with several diseases

Agler et al. reported that a mutation in RAB24 is associated with canine ataxia, a hereditary neurodegenerative disease.[55] The observed mutation results in glutamine to proline change in amino acid 38, located in the putative switch I region of RAB24. Glutamine 38 is well conserved in RAB24 in different species, and it is possible that the Q38P mutation has an effect on nucleotide binding. Affected dogs exhibit Purkinje neuron loss in the cerebellar cortex. Immunohistochemistry showed accumulation of ubiquitin-positive bodies in cells of the granular layer and at the junction of molecular and granular layers, and electron microscopy revealed axonal spheroids containing numerous late autophagic vacuoles in Purkinje cells of the granular layer. This study[55] is well in agreement with our findings, showing that nucleotide binding is important for the recruitment of RAB24 to autophagic compartments and that RAB24 is needed for autolysosome clearance.[42]

Altered expression of RAB24 has been reported in several human diseases, but further studies are needed to clarify whether the changes in RAB24 expression level are connected with alterations in autophagic activity. It is also unclear whether the altered RAB24 expression is a cause or consequence of the disease. Swaminathan et al. studied the mRNA levels of 59 selected genes between symptomatic patients (unstable plaques) and asymptomatic patients (stable plaques) suffering from carotid atherosclerosis.[56] LC3B showed the highest fold difference between the two groups: mRNA and proteins levels of LC3 were significantly decreased in the symptomatic samples. RAB24 mRNA was also significantly decreased in the symptomatic samples. Igci et al. studied gene expression profiles of autophagy-related genes in multiple sclerosis, an inflammatory disease of the central nervous system.[57] The expression of several genes, including RAB24, was observed to be altered (increased or decreased) in the patient samples. Jenum et al. aimed to find diagnostic biomarkers for pediatric tuberculosis.[58] They analyzed mRNA levels both direct ex-vivo, and using in

vitro whole blood stimulated with bacteria. They identified several biomarkers consistently associated with tuberculosis infections, one of them being RAB24. Elevated RAB24 mRNA levels were significantly associated with culture-positive tuberculosis.

Chen et al. investigated epigenetic silencing of micro RNA in hepatocellular carcinoma (HCC) and observed miR-615-5p to be downregulated in HCC.[59] Further, miR-615-5p was found to downregulate RAB24, while low levels of miR-615-5p increased the expression of RAB24 and facilitated the growth and metastasis of HCC both in vitro and in vivo. Downregulation of miR-615-5p and upregulation of RAB24 promoted the epithelial-mesenchymal transition, adhesion and vasculogenic mimicry of HCC cells. All these features enhance metastasis. Thus, RAB24 is a direct target of miR-615-5p, and RAB24 protein promotes the malignant phenotype of HCC cells. The authors concluded that miR-615-5p functions as a tumor suppressor by inhibiting RAB24 expression in HCC. These findings are in line with a report showing that RAB24 is required for normal cell division, modulating several mitotic events including chromosome segregation and cytokinesis.[60] It is currently unclear whether the roles of RAB24 in metastasis and cytokinesis are connected with its functions in autophagy or endocytosis.

Putative RAB24 effectors implicate a role in membrane fusion events

RAB24 effectors are at present unknown, but several studies support the hypothesis that RAB24 may function in membrane fusion. As mentioned earlier, Amaya et al.[54] reported that RAB24 coprecipitated with RAB7 and its effector RILP. Both RAB7 and RILP are known to function in endosome-lysosome fusion. Further, Schardt et al. found that RAB24 coprecipitated with synaptosomal associated proten 29 (SNAP29).[61] SNAP29 interacts with several syntaxins, SNARE proteins that participate in exocytosis.[62] The interaction of RAB24 with SNAP29 did not require the presence of GTPγS, unlike the interaction of SNAP29 with RAB3A.[61] Interestingly, SNAP29 was shown to play a role in autophagosome fusion with endosomes or lysosomes, acting in a SNARE complex with STX17/syntaxin 17.[63-66] Unlike RAB24, SNAP29 seems to be required for both basal and starvation-induced autophagy. Further, double-membrane autophagosomes accumulate in cells deficient in SNAP29[65] or STX17,[66] whereas we found that single-membrane bound, acidic and degradative autophagic vacuoles/autolysosomes accumulate in RAB24 deficient cells.[42]

Behrends et al. used HA-tagged RAB24 as one of the bait proteins in their proteomics study on interactions of autophagy proteins.[41] Their mass spectrometry primary data showed coprecipitation of RAB24 with GDP dissociation inhibitors 1 and 2 (GDI1 and GDI2), N-ethylmaleimide sensitive fusion protein (NSF), and plakophilin 1 (armadillo repeat protein implicated to function in desmosomes). Behrends et al. also found RAB24 among the proteins that coprecipitated with the SNARE protein Golgi SNAP receptor complex member 1 (GOSR1), but no coprecipitation was reported between RAB24 and SNAP29. In order to identify putative high-confidence interaction partners, Behrends et al. performed a comparative analysis of the proteomic results, and a subsequent analysis to validate and delineate the interaction network. This analysis placed RAB24 in the NSF subnetwork together with GOSR1, SNAP29 and several other SNARE proteins. The analysis proposes that RAB24 has direct interactions with GDI1, GDI2, NSF and plakophilin 1, while NSF would mediate the interactions with the other proteins in the subnetwork. Taken together, the findings of Behrends et al. support the idea that RAB24 may function in membrane fusion together with NSF, SNAP29 and GOSR1. Interaction of RAB24 with GDIs is expected, while the significance of the interaction with plakophilin 1 remains unknown.

Several laboratories have reported single RAB24 interacting proteins, but the importance of these interactions is not known at present. Tambe et al. observed that RAB24 coprecipitated with the tumor suppressor protein DRS.[48] Schlager et al. performed a GST pulldown assay and observed that, similar to several other RABs, RAB24 weakly bound Bicaudal-D-related protein 2/BICDR2 (a putative RAB6 effector).[67] Fukuda et al. used yeast two hybrid assay and immunoprecipitation to show that two mutant versions of RAB24 (S67L and T21N) interacted with transcriptional corepressor C-terminal-binding protein 1, CtBP1.[68]

In summary, several of the putative indirect or direct RAB24 interacting proteins have been implicated in membrane fusion, including RAB7, SNAP29, GOSR1 and NSF. The candidate RAB24 interactors are in agreement with the idea that RAB24 functions in membrane fusion events during the late steps of macroautophagic and endocytic pathways. However, further studies are required to elucidate the detailed molecular mechanisms of RAB24 functions.

Acknowledgments

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480 Figure legends

481

482 Figure 1. The RAB activation/inactivation cycle. RAB proteins cycle between active membrane
483 bound state and inactive cytosolic state. RABs recruit effector proteins while in the active GTP-
484 bound state (left). See text for further details. GEF, guanine nucleotide exchange factor; GAP,
485 GTPase activating protein; GDI, GDP dissociation inhibitor; GDF, GDI displacement factor; Pi,
486 inorganic phosphate; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein
487 receptor; t-SNARE, SNARE on target membrane; v-SNARE, SNARE on vesicle membrane.

488

489 Figure 2. There are three types of autophagy: microautophagy, chaperone-mediated autophagy
490 and macroautophagy. See text for further details.

491

492 Figure 3. RAB24 colocalizes with the autophagosome marker LC3. HeLa cells were transfected with
493 RAB24 and immunolabeled with anti-RAB24 and anti-LC3. Before fixation, the cells were treated
494 with 100 mM leupeptin and 10 mg/ml pepstatin for 6 h in full culture medium in order to accumulate
495 autophagic vacuoles (autophagosomes, amphisomes and autolysosomes) under basal conditions.
496 Yellow color in the overlay images indicates colocalization.